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Am J Physiol Endocrinol Metab, September 1, 2004; 287 (3): E558-E565.

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Intramyocellular lipids form an important substrate source during moderate intensity exercise in endurance-trained males in a fasted state

L. J. C. van Loon, R. Koopman, J. H. C. H. Stegen, A. J. M. Wagenmakers, H. A. Keizer and W. H. M. Saris

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Influence of prolonged endurance cycling and recovery diet on intramuscular triglyceride content in trained males

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Van Loon, Luc J. C., Vera B. Schrauwen-Hinderling, René Koopman, Anton J. M. Wagenmakers, Matthijs K. C. Hesselink, Gert Schaart, M. Eline Kooi, and Wim H. M. Saris. Influence of prolonged endurance cycling and recovery diet on intramuscular triglyceride content in trained males. *Am J Physiol Endocrinol Metab* 285: E804–E811, 2003. First published June 3, 2003; 10.1152/ajpendo.00112.2003.—Intramuscular triglycerides (IMTG) are assumed to form an important substrate source during prolonged endurance exercise in trained males. This study investigated the effects of endurance exercise and recovery diet on IMTG content in vastus lateralis muscle. Nine male cyclists were provided with a standardized diet for 3 days, after which they performed a 3-h exercise trial at a 55% maximum workload. Before and immediately after exercise and after 24 and 48 h of recovery, magnetic resonance spectroscopy (MRS) was performed to quantitate IMTG content. Muscle biopsies were taken after 48 h of recovery to determine IMTG content by using quantitative fluorescence microscopy. The entire procedure was performed two times; in one trial, a normal diet containing 39% energy (En%) as fat was provided (NF) and in the other a typical carbohydrate-rich athlete's diet (LF: 24 En% fat) was provided. During exercise, IMTG content decreased by $21.4 \pm 3.1\%$. During recovery, IMTG content increased significantly in the NF trial only, reaching preexercise levels within 48 h. In accord with MRS, fluorescence microscopy showed significantly higher IMTG content in the NF compared with the LF trial, with differences restricted to the type I muscle fibers (2.1 ± 0.2 vs. $1.4 \pm 0.2\%$ area lipid staining, respectively). In conclusion, IMTG content in the vastus lateralis muscle declines significantly during prolonged endurance exercise in male cyclists. When a normal diet is used, IMTG contents are subsequently replenished within 48 h of postexercise recovery. In contrast, IMTG replenishment is impaired substantially when a typical, carbohydrate-rich athlete's diet is used. Data obtained by quantitative fluorescence microscopy correspond well with MRS results, implying that both are valid methods to quantify IMTG content.

muscle; nuclear magnetic resonance; ¹H magnetic resonance spectroscopy; oil red O; exercise; intramyocellular lipid; diet

FAT AND CARBOHYDRATE are the principal substrates that fuel aerobic ATP synthesis in skeletal muscle. During

moderate-intensity exercise, fat oxidation contributes about one-half of total energy expenditure, with plasma free fatty acids (FFA) as the main fat source (38, 45, 55). Recently, we quantitated FFA oxidation rates in trained male cyclists during moderate-intensity exercise, which represented ~50% of total fat oxidation (55). Other fat sources contributing to total fat oxidation include plasma lipoprotein-derived triglycerides and intramuscular triglyceride (IMTG) stores. With the contribution of lipoprotein-derived triglyceride oxidation generally assumed to be of little quantitative importance (22, 39), IMTG stores are likely to form a major substrate source during exercise. However, the latter assumption has been disputed, as studies applying direct triglyceride measurements from muscle biopsies collected before and after exercise have reported contradicting results (57). This apparent controversy likely results from the marked between-biopsy variability in IMTG content when this extraction method is used (57).

With the introduction of magnetic resonance spectroscopy (MRS), a noninvasive means to quantitate muscle lipid content has become available (8). Several groups have applied MRS to quantitate IMTG content in various populations, which has led to the proposed relationship among increased IMTG content, obesity, and decreased insulin sensitivity (19, 28, 33, 41). The latter has strongly renewed interest in the role of exercise and diet in the regulation of IMTG storage and utilization. So far, several studies have used MRS to assess IMTG content before and after prolonged endurance exercise (5, 9, 10, 15, 34, 36, 43, 48). All of them have reported substantial (~20–40%) decreases in IMTG content after prolonged exercise. In most of these studies, IMTG content was determined by performing MRS on lower leg muscle (tibialis anterior and posterior and/or soleus muscle). The latter is in clear contrast to the vast majority of IMTG utilization studies that applied the triglyceride extraction method on muscle biopsies taken from the vastus lateralis. Clearly, MRS studies assessing IMTG content in the

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Table 1. *Subject characteristics*

Age, yr	23.9 ± 0.8
Body weight, kg	72.7 ± 1.3
Height, m	1.82 ± 0.02
BMI, kg/m ²	21.9 ± 0.6
W _{max} , W	421 ± 11
W _{max} , W/kg	5.8 ± 0.1
$\dot{V}O_{2max}$, l/min	5.0 ± 0.2
$\dot{V}O_{2max}$, ml·min ⁻¹ ·kg ⁻¹	68.1 ± 1.9
Maximal heart rate, beats/min	197 ± 1

Data are means ± SE; *n* = 9 subjects. BMI, body mass index; W_{max}, maximal workload; $\dot{V}O_{2max}$, maximal oxygen uptake.

vastus lateralis after various exercise and/or dietary interventions are warranted.

Because most exercise studies have focused on IMTG depletion, far fewer data are available on postexercise IMTG repletion (14, 15, 36). The latter is in contrast to the numerous studies on muscle glycogen use and the efficacy of dietary interventions to accelerate muscle glycogen synthesis (27), which have led to the recommendation for endurance athletes to use high-carbohydrate, low-fat diets (42a). Interestingly, recent MRS data have shown that the use of (extremely) low-fat diets can substantially impair postexercise IMTG repletion (14, 15, 36). In those studies, fat intake was set as low as 10–15% of total energy intake (En%) in the low-fat trials, which is considerably less than the reported fat intake in elite endurance athletes (23 En%; see Ref. 46). The latter, of course, is still well below fat intake in a normal Western diet (~35–40 En%; see Ref. 16). We questioned whether a common carbohydrate-rich athlete's diet limits postexercise IMTG repletion when compared with a normal diet.

MRS provides information on mixed-muscle IMTG content but does not discriminate between muscle fiber type-specific IMTG. In endurance-type exercise activities, muscle fiber recruitment mainly involves the use of type I muscle fibers (24). We recently optimized the use of fluorescence microscopy to quantify IMTG content at the muscle fiber level (32) and observed a substantial decrease in IMTG content after 2 h of endurance exercise in trained male athletes, which was shown to be restricted to the type I muscle fibers (54). Therefore, it would be important to determine whether differences in IMTG content after postexercise recovery on a normal or carbohydrate-rich athlete's diet are specific for muscle fiber type. In this study, we aimed to determine IMTG content in the vastus lateralis of male cyclists before and after prolonged endurance cycling. In addition, we investigated the time course of subsequent IMTG repletion after 2 days of postexercise recovery while a normal diet or a typical carbohydrate-rich athlete's diet was used. In addition to these repeated MRS measurements, muscle biopsies from the vastus lateralis were collected after 2 days of recovery to assess muscle fiber type-specific IMTG content by applying quantitative fluorescence microscopy on oil red O-stained muscle cross sections.

METHODS

Subjects. Nine male endurance-trained cyclists participated in this study. Subject characteristics are shown in Table 1. All subjects were informed about the nature and putative risks of the experimental procedures before their informed consent was obtained. This study was approved by the local Medical Ethics Committee.

Pretesting. Maximum oxygen uptake ($\dot{V}O_{2max}$) and maximum workload (W_{max}) were measured on an electronically braked cycle ergometer (Lode, Groningen, the Netherlands) during an incremental exhaustive exercise test 2 wk before the first experimental trial (35). Findings were used to determine the 55% W_{max}, incorporated in the exercise trials. The 55% W_{max} has been observed earlier to result in maximal absolute fat oxidation rates in trained male cyclists (55).

Standardization diet. All subjects received a standardization diet (30 En% fat) during the 3 days preceding both exercise trials, which were performed in the afternoon of the 3rd day. Subjects were provided with a standard package of food products, beverages, and instant meals and were allowed to use these ad libitum. All main meals (breakfast, lunch, and dinner) and between-meal snacks were instructed to be taken at predetermined time intervals during each day. Total energy intake and macronutrient composition of actual dietary intake were calculated from the provided package minus the amount of food that was left from each package (Table 2). Subjects were instructed to refrain from heavy physical labor and/or exercise during both standardization periods.

Protocol. On the 3rd day of both standardization diets, subjects came to the laboratory at 2:00 PM. First, an MRS scan was performed to determine preexercise IMTG content. Thereafter, a blood sample was obtained from an antecubital vein. After 5 min of warming up at 100 W, subjects started cycling at a 55% W_{max} for a 3-h period. Gas exchange measurements were performed each half-hour over 10-min periods (Oxycon β; Mijnhardt, Mannheim, Germany) to determine total fat and carbohydrate oxidation rates (40, 55). Subjects were allowed to drink water ad libitum during their first exercise trial, and the same amount of water was provided during their second trial. In case of the inability to maintain the 55% W_{max} during the latter stages of the exercise trials, workload was decreased to 45% W_{max}. After cessation of exercise, subjects were allowed a quick shower (maximum 5 min), after which a blood sample was collected and a second MRS scan was performed. Subsequently, the athletes were subjected to 2 days of either a carbohydrate-rich, low-fat diet (LF) or a normal-fat-containing diet (NF). Similar to the standardization diets, subjects were provided with (LF or NF) packages of food products, beverages, and instant meals and allowed to use these ad libitum. Dinners (evening meals) were all eaten under supervision at the

Table 2. *Dietary intake before exercise and during recovery*

Dietary Intake	Standardization Preexercise	Low-Fat Recovery	Normal-Fat Recovery
Energy intake, MJ/day	14 ± 0.2	14 ± 0.5	15 ± 0.4
Fat intake, En%	30 ± 0.3	24 ± 1.7	39 ± 0.3*
Carbohydrate intake, En%	56 ± 0.8	62 ± 2.5	49 ± 0.3*
Protein intake, En%	13 ± 0.2	14 ± 0.3	14 ± 0.1

Data are means ± SE. En%, total energy intake. *Significant difference in the contribution of fat and carbohydrate to total energy intake between the low-fat and normal-fat diets.

laboratory after the second and third MRS scan. After finishing their LF or NF meal, subjects left the laboratory to return after 24 and 48 h for another MRS scan, blood sample, and subsequent dinner. In addition, after the fourth MRS scan (48 h postexercise), a muscle biopsy was collected from the vastus lateralis of the same leg that was measured in the multiple MRS scans.

Intervention diets. Subject performed two exercise trials, after which different recovery diets were provided (the LF and NF diets). Food packages and evening dinners in both diets were designed to result in an estimated fat content between 20 and 25 and 35 and 40 En%, respectively. The LF and NF dietary intervention protocols were performed in a randomized order and were separated by a period of at least 3 wk.

MRS. Image-guided, localized, single-voxel ^1H -MRS was performed in the vastus lateralis. All measurements were performed on a 1.5-T whole body scanner (Intera; Philips Medical Systems, Best, the Netherlands) with a flexible surface coil wrapped around the upper leg with the leg placed in the parallel position. In every subject, voxels were carefully placed at the same position for all eight measurements. Care was taken to avoid vascular structures and/or adipose tissue within the voxel. To reproduce the same voxel position, longitudinal distance from the voxel to the intercondylar eminence of the knee joint was determined in a coronal image of the upper leg. The second relaxation time (T_2)-weighted turbo spin-echo MR images, consisting of five transversal slices, were acquired at this position [repetition time/echo time (TR/TE), slice thickness 5 mm, 2,000/85 ms, echo train length 12, field of view 210 mm, and matrix size 256×256]. The patterns of fat distribution were used to verify the longitudinal position and as landmarks to reproduce the voxel position in the transversal plane.

^1H -MRS spectra from the regions of interest were acquired using a point-resolved spectroscopy sequence with the following acquisition parameters: TR/TE 3,000/25 ms, 16 phase cycles, 128 averages, and 1,024 data points over 1,000 Hz spectral width. The voxel volume was $12 \times 11 \times 18 \text{ mm}^3$. The water signal was suppressed using Chemically Selective Saturation. The unsuppressed water signal was measured subsequently in the same voxel under the same shimming conditions and used as a reference. For postprocessing, the spectra were fitted in the time domain using a nonlinear least squares algorithm (52) in the jmrui software package. Three peaks were fitted for intramyocellular (IMCL) and three peaks for extramyocellular (EMCL) lipids. Prior knowledge (9, 53) of the relative peak positions (9, 53) and area ratios as determined by Boesch C and Kooi ME (personal communications) were used as a constraint. To increase accuracy and reliability of the fitting procedure, the line width of the CH_2 peak of IMCL lipids was fixed relative to the line width of the unsuppressed water peak (in the same voxel), and soft constraints were used to limit the line width and position of the CH_2 peak of EMCL relative to that of the IMCL. IMCL and EMCL peaks were fitted with a Gaussian curve, while water peaks were fitted with a Lorentzian curve. The signals were corrected for T_1 and T_2 relaxation using the T_1 and T_2 relaxation times as determined by Schick and colleagues (5, 9, 47, 51). The corrected area of the CH_2 peak of IMCL was expressed relative to the area of the water peak. Similar findings, with a greater coefficient of variance, were obtained when expressed relative to the creatine peak. Figure 1 shows a typical MRS acquired in the present study, showing EMCL signals to be relatively small in the vastus lateralis of these trained cyclists. The reproducibility of the IMCL quantification in the vastus lateralis was determined by measuring one

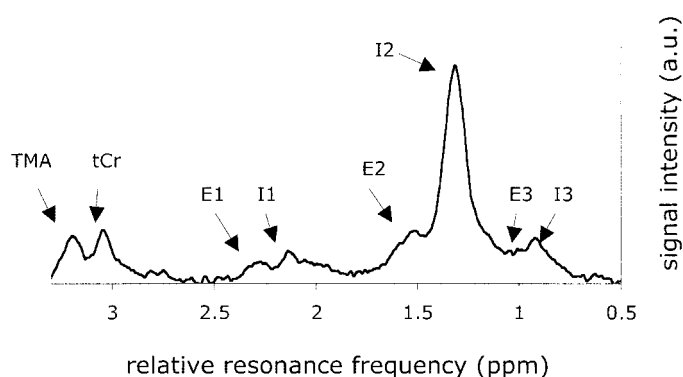


Fig. 1. Typical spectrum acquired using image-guided, localized single-voxel ^1H magnetic resonance spectroscopy (MRS) in the vastus lateralis. TMA, trimethyl ammonium compounds; tCr, total creatine; E1, broad peak of various chemically different methylene protons of extramyocellular (EMCL; see Ref. 7) lipids; E2, methylene protons of EMCL; E3, methyl protons of EMCL; I1, broad peak of various chemically different methylene protons of intramyocellular (IMCL; see Ref. 7) lipids; I2, methylene protons of IMCL; I3, methyl protons of IMCL; AU, arbitrary units.

subject two times on several occasions. The subject left the scanning table between measurements, after which the coil was repositioned. The comparison of the IMCL quantification in corresponding voxels ($n = 5$) resulted in a coefficient of variation of $5.0 \pm 3.3\%$, which is in accord with earlier reports on the obtained variation when measuring IMTG content by using MRS (5, 9, 47, 51).

Blood and muscle tissue analysis. Blood was collected in EDTA-containing tubes and centrifuged at 1,000 g and 4°C for 5 min. Aliquots of plasma were frozen immediately in liquid nitrogen and stored at -80°C . Plasma FFA (Wako NEFA-C kit; Wako Chemicals, Neuss, Germany), free glycerol (148270; Roche Diagnostics, Indianapolis, IN), and triglyceride (GPO-trinder 337B; Sigma Diagnostics, St. Louis, MO) concentrations were analyzed using the COBAS FARA semiautomatic analyzer (Roche, Basel, Switzerland).

Muscle samples were dissected carefully, freed from any visible nonmuscle material, rapidly frozen in liquid nitrogen-cooled isopentane, and embedded in Tissue-Tek (Sakura Finetek, Zoeterwoude, The Netherlands). Multiple serial sections (5 μm) from biopsies collected in both trials were thaw mounted together on uncoated, precleaned glass slides. To permit quantification of IMCL stained by oil red O together with immunolabeled cellular constituents, we used the protocol described by Koopman et al. (32). Briefly, cryosections were fixed in 3.7% formaldehyde for 1 h. Slides were rinsed with deionized water, treated with 0.5% Triton X-100 in PBS, and washed with PBS. Thereafter, sections were incubated with antibodies against human laminin (polyclonal rabbit antibody; Sigma Diagnostics, Steinheim, Germany) and human myosin heavy chain (A4.840), developed by Dr. H. M. Blau (12), enabling us to visualize individual cell membranes and to determine muscle fiber type (I or II), respectively. Incubation was followed by washes in PBS, after which the appropriate conjugated antibodies GARiGg, Alexa350, and GAMiGm Alexa488 (Molecular Probes, Leiden, The Netherlands) were applied. After several washes with PBS, glass slides were immersed in the oil red O working solution. Oil red O stock solution was prepared by adding 500 mg of oil red O (Fluka Chemie, Buchs, Switzerland) to 100 ml 60% triethyl phosphate. Before staining, a 36% triethyl phosphate working solution, containing 12 ml of oil red O stock solution and 8 ml of deionized water, was prepared and filtered to remove

crystallized oil red O. After 30 min of oil red O immersion, slides were rinsed with deionized water followed by a 10-min wash with tap water. Stained sections were embedded in Mowiol and covered with a coverslip. After 24 h, slides were examined using a Nikon E800 fluorescence microscope (Uvikon; Bunnik, The Netherlands) coupled to a Basler A113 C progressive scan color CCD camera, with a Bayer color filter. Epifluorescence signal was recorded using a Texas red excitation filter (540–580 nm) for oil red O, a DAPI UV excitation filter (340–380 nm) for laminin, and an FITC excitation filter (465–495 nm) for muscle fiber type. Multiple digitally captured images ($\times 40$ objective), at least five fields of view per muscle cross section, were processed and analyzed using Lucia 6.01 software (Nikon, Düsseldorf, Germany). The oil red O epifluorescence signal was quantified for each cell, within five fields of view for each muscle section. An intensity threshold representing minimal absorbance values corresponding to lipid droplets was set manually and used for all images. Total area measured and the area and number of objects emitting oil red O epifluorescence signal were recorded. Fiber type-specific IMTG content was expressed as the percentage of the measured area that was stained with oil red O in each muscle cell. Average lipid droplet size was calculated by dividing the total area of lipid stained by the total number of droplets. Lipid droplet density was calculated by dividing the total number of droplets by the total (IMCL) area measured.

Statistics. All results are expressed as means \pm SE. Statistical significance of differences between trials was assessed by paired *t*-tests. A repeated-measures ANOVA was applied to assess statistical differences over time within trials. A Sheffé post hoc test was applied to locate any differences over time. Simple regression analysis was performed on mixed-muscle IMTG content, as determined by MRS, and average muscle fiber IMTG content, as determined by quantitative fluorescence microscopy (corrected for muscle fiber type composition). Statistical significance was set at $P < 0.05$.

RESULTS

Exercise trials. In the exercise trials, subjects cycled for 3 h at an average workload of 232 ± 6 W (55% W_{\max}). Because of fatigue, power output was decreased to 45% W_{\max} in two subjects during the last 30 min of the 3-h exercise period. The same procedure was repeated in the second trial. The 55% W_{\max} corresponded to $62 \pm 1\%$ $\dot{V}O_{2\max}$. Substrate use and total energy expenditure were similar in both exercise trials and are reported in Table 3. MRS quantification of IMTG content before and after the exercise trials is shown in Fig. 2. IMTG content was decreased significantly by

Table 3. Substrate use during the exercise trials before dietary intervention

	Low-Fat Diet (<i>n</i> = 9)	Normal-Fat Diet (<i>n</i> = 9)
Energy expenditure, MJ	11.7 ± 0.21	11.7 ± 0.28
Fat oxidation, g	100 ± 7	88 ± 3
Carbohydrate oxidation, g	469 ± 20	502 ± 20
Fat oxidation, En%	35 ± 2	31 ± 1
Carbohydrate oxidation, En%	65 ± 2	69 ± 1

Data are means \pm SE; *n*, no. of subjects. There were no significant differences between trials.

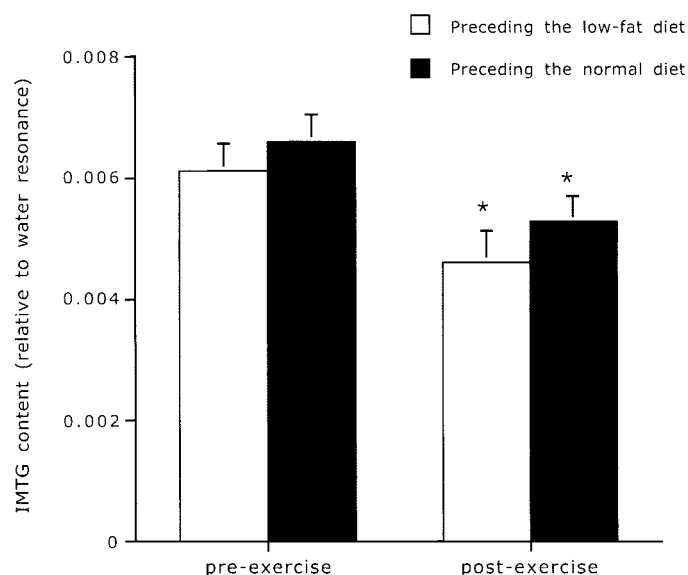


Fig. 2. Intramuscular triglyceride (IMTG) content (relative to water resonance) before and after exercise as quantitated by MRS. Data are expressed as means \pm SE. *Significantly decreased compared with preexercise values ($P < 0.05$).

22.6 ± 5.3 and $20.4 \pm 3.7\%$ in both exercise trials preliminary to the LF and NF trials, respectively ($P < 0.05$). No differences were observed between trials.

Diet composition. Dietary intake during the 3-day standardization period (before the exercise trials) and subsequent dietary intake in the 2-day LF and NF diets are reported in Table 2. Dietary intake during both standardization periods was similar. In the recovery period, total energy and protein intake were not different between trials. However, fat and carbohydrate intake differed significantly; 24 vs. 39 En% fat and 62 vs. 49 En% carbohydrate in the LF and NF trials, respectively ($P < 0.05$).

Plasma analysis. Plasma FFA, free glycerol, and triglyceride concentrations are provided in Table 4. Plasma FFA and glycerol concentrations were increased significantly after exercise. After 24 and 48 h of recovery, concentrations were similar to preexercise values. Plasma triglyceride concentrations were reduced substantially after exercise and remained below baseline levels after 24 h of postexercise recovery. At 48 h postexercise, plasma triglyceride concentrations had increased significantly and were similar to preexercise levels. No significant differences were observed between trials.

IMTG repletion. In the postexercise recovery period, IMTG content was increased significantly in the NF trial only ($P < 0.05$), with post hoc analysis showing a significantly higher IMTG content after 48 h compared with the values reported immediately and 24 h after exercise. In the LF trial, no significant changes in IMTG content were observed (Fig. 3). Comparison of preexercise IMTG content with values recorded after 48 h of recovery showed similar content in the NF trial and a significantly lower IMTG content after 48 h of recovery in the LF trial.

Table 4. Plasma concentrations

	0 h	3 h	24 h	48 h
Free fatty acids				
Low-fat diet	104 ± 11	1,425 ± 59*†‡	197 ± 44	177 ± 42
Normal-fat diet	90 ± 12	1,400 ± 61*†	235 ± 38	317 ± 107
Free glycerol				
Low fat diet	58 ± 9	442 ± 43*†‡	67 ± 7	66 ± 8
Normal-fat diet	45 ± 5	469 ± 63*†	70 ± 6	65 ± 10
Plasma triglycerides				
Low-fat diet	1,384 ± 112	644 ± 52*‡	740 ± 129*‡	1,421 ± 149
Normal-fat diet	1,522 ± 189	652 ± 49*	750 ± 101*	1,222 ± 211

Data are means ± SE. Plasma free fatty acid, glycerol, and triglyceride concentrations before exercise (0 h), within 5 min after exercise (3 h), and 24 and 48 h postexercise. Plasma free fatty acid, glycerol, and triglyceride concentrations are expressed in $\mu\text{mol/l}$. *Significantly different from baseline value (0). †Significantly different from values at 24 h postexercise. ‡Significantly different from values at 48 h postexercise. There were no significant differences between trials.

In accord with the MRS data, muscle tissue analysis for IMTG quantification using quantitative fluorescent microscopy on oil red O-stained muscle cross sections showed significantly higher IMCL content in the type I fibers after 48 h of recovery in the NF compared with the LF trial (Table 5; $P < 0.05$). The higher lipid content was accounted for by a significantly greater lipid droplet size and an increased lipid droplet density. IMCL content in the type II fibers was not different between trials. Lipid content in the type II fibers was significantly lower compared with the type I fibers. The latter was accounted for by a lower lipid droplet density ($P < 0.05$) but not lipid droplet size (not significant).

To enable a direct comparison between the data on IMTG content in mixed muscle as determined by MRS and muscle fiber type-specific IMTG content as determined by quantitative fluorescence microscopy on oil red O-stained muscle cross sections, we corrected the latter for muscle fiber type composition (Table 5). Muscle fiber type composition of the multiple muscle cross

sections showed an average $58 \pm 4\%$ type I and $42 \pm 4\%$ type II fibers. Comparison of the data on (mixed-muscle) IMTG content after 48 h of postexercise recovery on both the LF and NF diets using both methods revealed a strong significant correlation ($R = 0.6$; $P = 0.01$).

DISCUSSION

In the present study, we observed a progressive increase in whole body fat oxidation rates during exercise, resulting in an average total of 94 ± 4 g of fat oxidized within the 3-h exercise trials (Table 3). Concomitantly, IMTG content in the vastus lateralis muscle, as measured by MRS, was reduced substantially by $21 \pm 3\%$ after exercise (Fig. 2). The observed decrease is in line with other MRS studies reporting similar (~ 20 – 40%) reductions in IMTG content in the tibialis anterior and soleus muscle before and after prolonged running or cycling exercise (5, 9, 10, 15, 34, 36, 43). We converted the IMTG data (Fig. 2) to absolute muscle concentrations using formulas and assumptions as published by Boesch et al. (5). Subsequently, IMTG contents before and after exercise in the vastus lateralis muscle averaged 7.9 ± 0.6 (pre) and 5.9 ± 0.7 (post) and 8.5 ± 0.6 (pre) and 6.8 ± 0.6 (post) mmol/kg wet

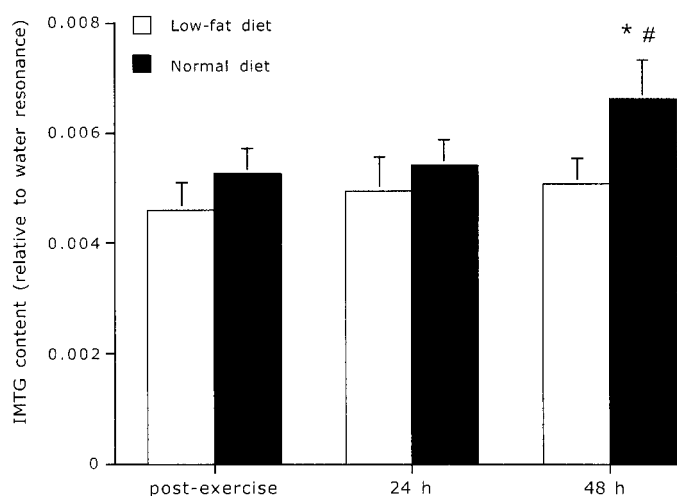


Fig. 3. IMTG (relative to water resonance) immediately after exercise and after 24 and 48 h of recovery on a low-fat or normal-fat-containing diet, as quantitated by MRS. Data are expressed as means ± SE. *Significantly higher compared with baseline (postexercise) value. #Significantly higher compared with the value observed after 24 h of recovery.

Table 5. Oil red O staining: muscle triglyceride content 48 h postexercise

	Low-Fat Diet (n = 9)	Normal-Fat Diet (n = 9)
<i>Type I fibers</i>		
IMTG content, %	1.39 ± 0.21	2.09 ± 0.24*
Droplet size, $\mu\text{m}^2/\text{droplet}$	0.87 ± 0.06	1.00 ± 0.04*
Droplet density, droplets/ μm^2	0.016 ± 0.002	0.020 ± 0.002*
Fiber type content, %	55 ± 7	60 ± 6
<i>Type II fibers</i>		
IMTG content, %	0.51 ± 0.11†	0.66 ± 0.12†
Droplet size, $\mu\text{m}^2/\text{droplet}$	0.84 ± 0.10	0.94 ± 0.07
Droplet density, droplets/ μm^2	0.010 ± 0.002†	0.009 ± 0.003†
Fiber type content, %	45 ± 7	40 ± 6

Data are means ± SE; n, no. of subjects. IMTG, intramuscular triglycerides. *Significantly higher in the normal-fat trial compared with the low-fat trial. †Significantly lower compared with the type I muscle fibers.



muscle, preceding the LF and NF diets, respectively. These absolute IMTG concentrations observed in the vastus lateralis are well above IMTG content reported for tibialis anterior, tibialis posterior, and/or soleus muscle (1, 2, 15, 26, 28, 29) and confirms that there are substantial differences in IMTG content between different muscle groups. The latter seems to be, at least partly, explained by differences in muscle fiber type composition (26).

Our data clearly show that IMTG stores in the vastus lateralis form an important substrate source during prolonged (cycling) exercise in male athletes. This confirms conclusions from stable isotope studies reporting that ~50% of total fat oxidation is accounted for by plasma FFA oxidation, suggesting that other substrate sources like IMTG play a substantial role in energy provision during exercise (22, 38, 45, 55). However, as noted in the introduction, the importance of IMTG stores as a substrate source has been disputed as some (4, 11, 13, 17, 20, 25, 42, 56), but certainly not all, studies (3, 21, 30, 31, 44, 49, 50, 58) using the biochemical triglyceride extraction method on muscle samples collected before and after exercise have been able to show a significant decline in IMTG content after prolonged endurance exercise. The apparent controversy, elegantly described by Watt et al. (57), can be explained partly by the marked between-biopsy variability (~24%) that has been reported when this technique (58) is used. Whether the high variability in muscle IMTG content is the result of the contamination of the obtained muscle samples with EMCL triglycerides (57, 58) and/or simply because of high variability in IMTG content between various muscle sections (50) is not clear (57). In accord with the apparent problems when using the biochemical triglyceride extraction method, Howald et al. (23) reported a good correlation between IMTG content in the tibialis anterior as determined by MRS and electron microscopy (EM), but the results from the muscle triglyceride extraction method did not correlate well with the results obtained by either EM or MRS.

In the postexercise phase, we observed a significant increase in IMTG content over the 48-h recovery period only when the NF diet was used. After 24 h of postexercise recovery, IMTG content was still low and did not differ from IMTG contents observed immediately after cessation of exercise (Fig. 2). Similarly, plasma triglyceride concentrations remained substantially lower compared with preexercise values (Table 4). After 48 h of postexercise recovery, IMTG content and plasma triglyceride levels had increased significantly on the NF diet. When IMTG content after 48 h of recovery on the NF diet was compared with the corresponding preexercise values, no significant differences were observed. Therefore, we conclude that, with a normal diet during recovery from prolonged endurance exercise, IMTG stores in the vastus lateralis can be replenished fully within 48 h. These findings seem to be somewhat different from those of Larson-Meyer et al. (36), who observed full restoration of the IMTG content in the soleus muscle of seven female endurance runners

within ~22 h of recovery from a 2-h treadmill run when using a similar normal-fat-containing diet (35 En%). The apparent discrepancy could likely be explained by a more extensive depletion of the IMTG stores in the vastus lateralis muscle after the 3-h cycling protocol in the present study. In addition, gender differences in IMTG use and/or storage (44, 50) and differences in the activation pattern of the different muscle groups during running or cycling are likely to contribute to the apparent differences in the observed time course of IMTG repletion.

In line with other studies on postexercise IMTG repletion (15, 36), we show that IMTG repletion is impaired substantially when a low-fat diet is used. In the present study, IMTG content did not increase during 48 h of postexercise recovery when a typical low-fat athlete's diet (24 En% fat) was used, with IMTG content still being significantly lower after 48 h of recovery compared with preexercise values ($P < 0.05$). In agreement, others have reported that ingestion of a low-fat diet does not allow IMTG content to return to preexercise levels within 30 (15), 22, and 70 (36) h of postexercise recovery. Although in those studies more extreme low-fat diets (10–15 En% fat) were applied, the present study shows that even a commonly used carbohydrate-rich athlete's diet containing ~24 En% fat (46) is insufficient to enable a full restoration of the IMTG stores within 48 h. As such, it could be speculated that such a dietary strategy potentially limits performance during periods of repeated bouts of prolonged endurance exercise. However, optimizing performance capacity by maximizing endogenous substrate repletion in the postexercise phase also includes the need to optimize postexercise muscle glycogen synthesis. Clearly, when fat intake is decreased to favor carbohydrate ingestion, IMTG restoration will become impaired. In reverse, when fat intake is favored to maximize IMTG repletion, muscle glycogen storage could prove to become suboptimal. Obviously, diets with an opposite fat-to-carbohydrate ratio seem to be preferred for IMTG or glycogen repletion, which makes the choice of postexercise dietary strategy controversial. As recently suggested by Décombaz et al. (15), a high-carbohydrate diet provided immediately postexercise followed by a normal- to high-fat diet could prove to optimize IMTG content without compromising muscle glycogen storage. More research is warranted to determine the best dietary strategy to optimize both IMTG and muscle glycogen storage.

The use of oil red O staining on muscle cross sections and subsequent quantification of the staining intensity by fluorescence microscopy have been optimized recently (32) and enable direct and fiber type-selective quantification of IMCL lipid content. Because of the invasiveness of the percutaneous muscle biopsies, muscle samples were only collected after 48 h of recovery in both trials. Similar to the data acquired by MRS, quantitative fluorescence microscopy showed substantially higher IMTG content after the use of the NF vs. the LF diet after 48 h of recovery. In agreement, a significant correlation was observed between the data

on (mixed-muscle) IMTG content obtained by the use of both methods ($R = 0.6$; $P < 0.01$). Subsequently, our data imply that both MRS and quantitative microscopy on oil red O-stained muscle cross sections represent valid methods to quantify IMTG content. With MRS being unable to provide information on fiber type-specific IMCL lipid content, we applied fluorescence microscopy to determine whether potential differences in IMTG content after the use of the NF and LF diet could be fiber type specific. Clearly, the higher mixed-muscle IMTG content after the NF vs. LF diet was explained by a significantly higher IMTG content in the type I but not the type II muscle fibers (Table 5). The latter is important, since muscle fiber type recruitment during endurance-type exercise predominantly relies on the use of type I muscle fibers (24), which have been shown to contain about three- to fourfold more lipid than type II fibers (18, 37). In agreement, we recently observed a substantial decrease in IMTG content, which was shown to be restricted to the type I muscle fibers, after prolonged (2-h) endurance exercise in trained male athletes (Van Loon LJC, Greenhaff PL, Constantin-Teodosiu D, Saris WHM, and Wagenmakers AJM, unpublished observations). Subsequently, the repletion of type I muscle fiber IMTG content could prove to be of great functional importance. More studies are warranted to provide detailed insight into muscle fiber type-specific IMTG utilization, the extent of IMTG depletion, and the time course of subsequent IMTG repletion during postexercise recovery.

In summary, the present study shows that IMTG content in the vastus lateralis muscle is reduced substantially after prolonged endurance cycling exercise in trained male athletes. During postexercise recovery, IMTG repletion is impaired substantially when a carbohydrate-rich athlete's diet is used, which is typically low in fat. As such, IMTG stores are not replenished within 48 h, which is in contrast to the situation when an NF diet is used. These results imply that carbohydrate-rich diets, as generally recommended to endurance athletes, are insufficient in providing ample substrate for IMTG repletion in male athletes. The latter could prove to result in suboptimal performance capacity during periods in which prolonged endurance exercise trials are performed repeatedly. In addition, data on IMTG content as obtained by using quantitative fluorescence microscopy on oil red O-stained muscle cross sections correspond well with MRS results, implying that both are valid methods to quantify IMCL lipid content.

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DISCLOSURES

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